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TITLE: Novel Role of Merlin Tumor Suppressor in Autophagy and its Implication in Treating NF2-Associated Tumors

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**14. ABSTRACT**
We have recently demonstrated that Merlin regulates the induction of autophagy, a cellular catabolic pathway implicated in the maintenance of cellular homeostasis. Deregulated autophagy is linked to a number of human disease conditions, including tumorigenesis. During this grant award period, we demonstrated that Merlin interacts with several autophagy-related proteins (i.e., LC3, Ulk1, and dynein), and LC3-Merlin-dynein complex formation was dependent on Ulk1 kinase activity. In addition, cell biological analyses showed that autophagy induction is attenuated in cells that express Merlin/K79E, a point mutation found in NF2 patients. Merlin K79E abolished the affinity to LC3. Additional Merlin mutants also showed attenuated affinity to LC3 and reduced ability to induce autophagy. Finally, we established the 3D culture system to evaluate the extent of metabolic stress caused by loss of Merlin function. We showed that loss of Merlin leads to attenuated autophagy and consequent elevation of metabolic stress, a condition known to accelerate tumor formation in vivo. This metabolic stress can be suppressed by an autophagy inducer rapamycin. These results suggest that autophagy induction can be an alternative avenue for treating NF2.

**15. SUBJECT TERMS**
Autophagy induction, metabolic stress, Ulk1/Atg1, rapamycin

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## Table of Contents

**Introduction:**

During the grant period, we tested our hypothesis that the tumor suppressor Merlin suppresses tumorigenesis in part by activating autophagy, and that this new role of Merlin in autophagy could be a target for therapeutic intervention against NF2. Autophagy is a cellular clearance system responsible for removing old proteins and damaged organelles within cells, and thus helps mitigate a risk of tumor formation. Our biochemical experiments demonstrated that Merlin is a component of machinery responsible for autophagosome formation. Our cell biological experiments revealed that loss of Merlin leads to attenuated autophagy, and that several Merlin mutations found in NF2 patients affect the autophagy-inducing activity of Merlin. In addition, we evaluated the effect of autophagy-inducing compounds in Merlin-deficient cell lines to prove that this strategy could effectively suppress cellular metabolic stress caused by loss of Merlin function. These analyses provide new insight into the avenue we could pursue to treat NF2-associated tumors.

**Body:**

Interaction studies of Merlin and autophagy-related proteins (Aim 1)

1a. Preparation of expression constructs

In our preliminary experiments aiming at identifying proteins that interact with Merlin tumor suppressor, we found Unc51.1/Atg1/Ulk1 as a binding partner for Merlin in the yeast two-hybrid screening. Unc51.1/Atg1/Ulk1 is an evolutionarily conserved regulator of autophagy that forms a large macromolecular complex upon autophagy induction. As a first step to study whether Merlin functions in autophagy, we characterized the Ulk1-Merlin interaction during autophagy induction. Merlin weakly bound Ulk1 under nutrient-rich conditions, and their interaction was transiently upregulated in response to nutrient starvation, showing a peak at around 10 min after autophagy induction, and rapidly downregulated by 30 min (**Figure 1**).

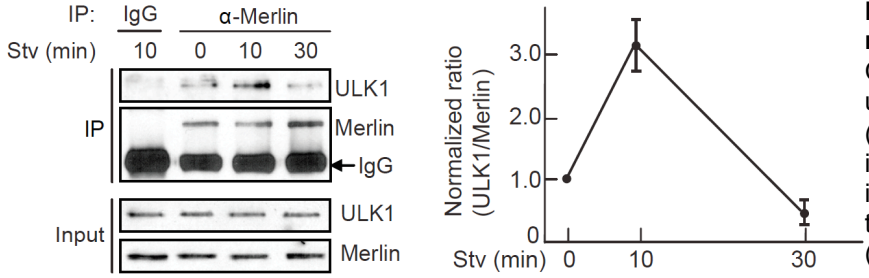



**Figure 1. Merlin interacts with Ulk1 in response to nutrient starvation**

Cell lysates of mouse embryonic fibroblasts were used for immunoprecipitation (IP) with anti-Merlin (α-Merlin) followed by Western blots using the indicated antibodies. Graph: The ratios of pixel intensity for Ulk1/Merlin bands were normalized to the value at the fed condition and plotted (mean±SEM: n=3).

To further investigate the role of Merlin in autophagy, we analyzed the interaction of Merlin with additional autophagy-related proteins, including LC3 and dynein intermediate chain (DIC). The autophagosome marker protein LC3 is reported to bind Ulk1, and Merlin contains multiple LC3-interacting regions (LIRs). DIC is a component of dynein motor complex responsible for autophagosome trafficking, and Merlin is reported to bind dynein. We constructed the mammalian expression plasmids necessary to carry out interaction studies, including GFP-tagged LC3, myc-tagged Merlin, HA-tagged Merlin, and myc-tagged Ulk1.

1b. Transfection and immunoprecipitation experiments

Using these reagents, we carried out immunoprecipitation experiments using HEK293T heterologous expression system. Merlin weakly bound LC3 at the fed condition, and their interaction was significantly upregulated upon autophagy induction (starvation 15min) (**Figure 2**). DIC co-precipitated with LC3 and Merlin upon autophagy induction, and the LC3−Merlin−DIC ternary complex formation was dependent on Merlin (**Figure 2**), suggesting that Merlin serves as an adaptor that links LC3 to dynein motor complex.

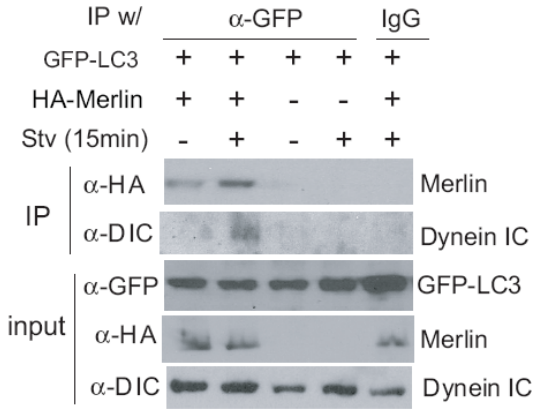



**Figure 2. Merlin interacts with LC3 and Dynein upon autophagy induction**

HEK293T cells were transfected with the plasmids expressing Merlin, LC3, or dynein intermediate chain (DIC). After autophagy induction by 15 min of nutrient starvation (Stv), the cell lysates were immunoprecipitated (IP) with anti-GFP (α-GFP) and analyzed by Western blots using the indicated antibodies.

Because Unc51.1/Atg1/Ulk1 is a kinase critical to autophagy induction, we tested whether the kinase activity influences the formation of LC3−Merlin−DIC complex. While the wild-type kinase markedly upregulated the complex formation, the dominant-negative form inhibited the complex formation (**Figure 3**), suggesting that Unc51.1/Atg1/Ulk1 kinase activity drives the trafficking of LC3−Merlin complex toward the retrograde direction.

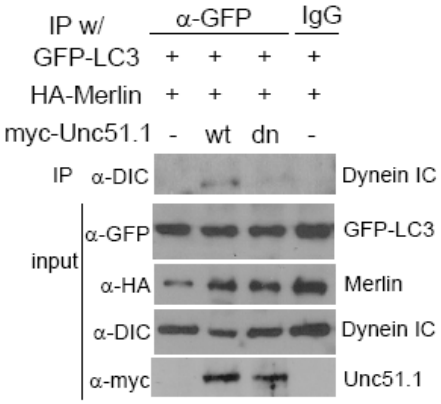



**Figure 3. Merlin−Dynein interaction is positively regulated by Unc51.1/Atg1/Ulk1 kinase activity**
HEK293T cells were transfected with the plasmids expressing Merlin, LC3, DIC, or Unc51.1 (wild-type [wt] or dominant-negative [dn]). The resulting cell lysates were immunoprecipitated (IP) with anti-GFP (α-GFP) and analyzed by Western blots.

On the contrary, the affinity of Merlin with kinesin light chain (Klc), a component of the kinesin anterograde motor, was inhibited by the Unc51.1/Atg1/Ulk1 kinase activity (**Figure 4**).

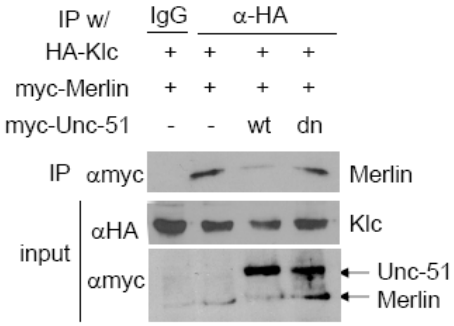
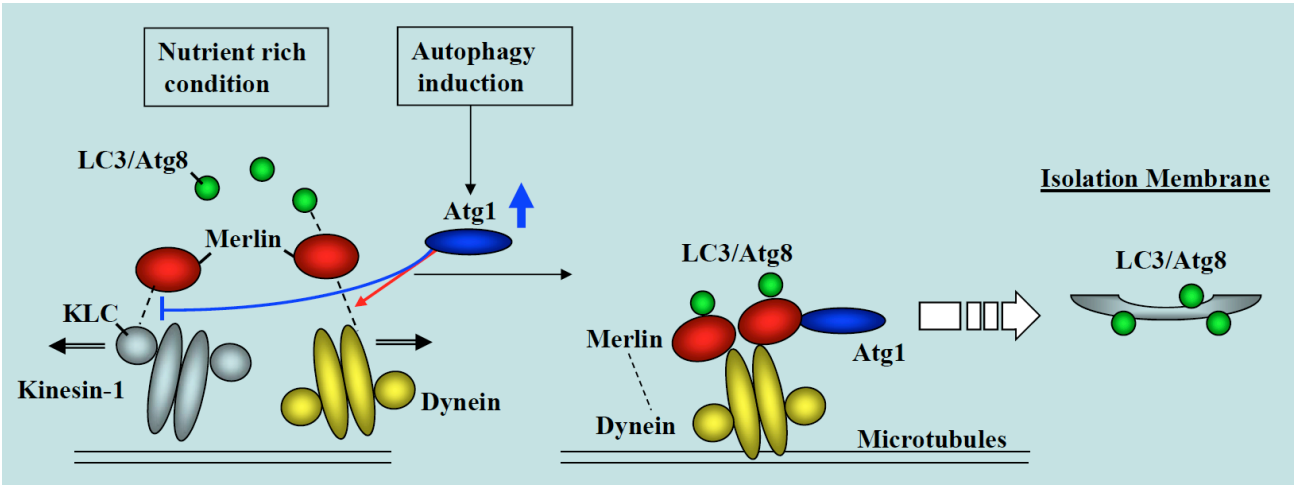



**Figure 4. Merlin−Kinesin interaction is negatively regulated by Unc51.1/Atg1/Ulk1 kinase activity**
HEK293T cells were transfected with the indicated plasmids and the resulting cell lysates were analyzed by immunoprecipitation (IP) with anti-HA (α-HA) or the control IgG, followed by Western blots using anti-myc (α-myc) and α-HA.

1c. Data analysis for 1a and 1b

Upon autophagy induction by nutrient starvation, Unc51.1/Atg1/Ulk1 kinase activity is upregulated and induces the association of Merlin and DIC, but inhibits the association of Merlin and kinesin light chain (Klc) (**Figures 1-4**). Based on these observations, we hypothesized that Merlin serves as an adaptor protein that links LC3 autophagy protein to dynein retrograde motor, thereby helping deliver LC3 towards the autophagic membranes.




1d. Setting up mouse crosses to prepare MEFs

*Unc51.1/Atg1/Ulk1* heterozygous mice were intercrossed and mouse embryonic fibroblasts (MEFs) were prepared from embryonic day 14 (E14) pups of wild-type and homozygous mice.

1e. Preparation of MEFs

*Unc51.1*(-/+);*GFP-LC3*/+ mice crossed with *Unc51.1*(-/+) mice provided GFP-positive MEFs and GFP-negative MEFs with an expected ratio (approx. 1:1). MEFs were prepared according to the standard procedure.

1f. Transfection of MEFs and interaction assays

Using MEFs prepared above, we carried out immunoprecipitation assays to confirm endogenous interaction among LC3, Merlin, and DIC. As a first step, the lentivirus constructs encoding Merlin-knockdown short-hairpin RNA (shRNA) were transfected to HEK293 cells together with the packaging plasmids to produce Merlin-knockdown lentiviruses. We then used these viruses to knockdown Merlin in MEFs. Merlin-knockdown significantly attenuated the complex formation between LC3 and DIC (**Figure 5**), suggesting that Merlin is a component of autophagy regulatory machinery.

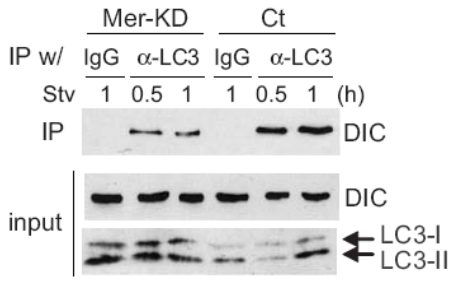



**Figure 5. Merlin knockdown attenuates the LC3–dynein complex formation in MEFs**
Merlin knockdown (Mer-KD) and control (Ct) MEFs were nutrient-starved for up to 1h to induce autophagy. The lysates were used for immunoprecipitation (IP) with anti-LC3 (α-LC3) followed by Western blots using the indicated antibodies.

To prove that Merlin regulates autophagy, we measured the autophagy flux in the presence or absence of Merlin. We confirmed that Merlin knockdown MEFs showed a significant decrease in the levels of autophagy flux, as evaluated under the treatment with lysosomal protease inhibitor Bafilomycin A1 (**Figure 6**), indicating that Merlin is critical to autophagy induction.

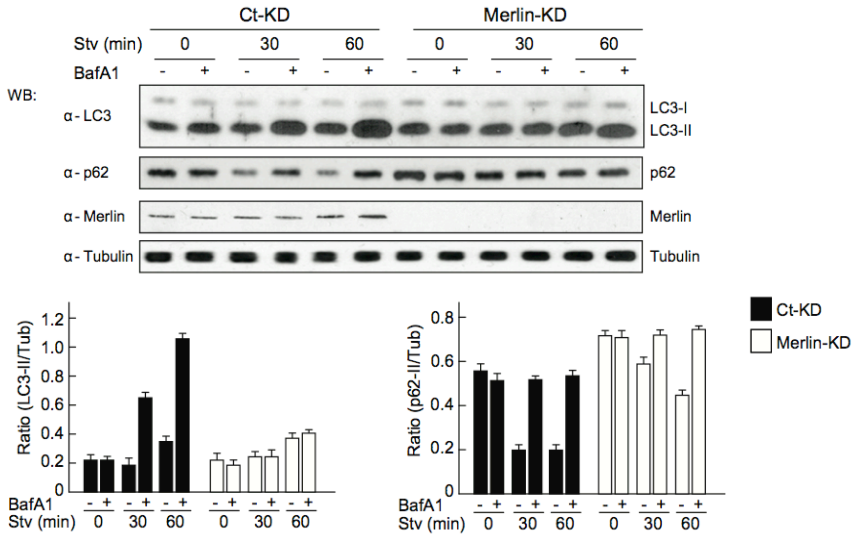



**Figure 6. Loss of Merlin leads to attenuated autophagy flux**
In response to nutrient starvation (Stv), Merlin-knockdown (KD) MEFs show attenuated levels of LC3-II accumulation or p62 degradation, as compared with control MEFs (Cr-KD). Graphs: Densitometric analysis of LC3-II and p62 on Western blots. Pixel intensities of LC3-II and p62 were normalized by those of α-Tubulin.

1g. Data analysis for 1f

As expected from the IP experiments using heterologous expression system (**Figures 1-4**), we showed the interaction of endogenous LC3 with DIC in MEFs. This interaction was significantly attenuated in Merlin-knockdown (KD) MEFs (**Figure 5**), consistent with the idea that Merlin serves as an adaptor linking LC3 to DIC. Moreover, Merlin-KD led to reduce levels of p62 degradation and LC3-II accumulation (**Figure 6**), suggesting that Merlin is a component of autophagy induction machinery, in which Merlin accelerates the integration of LC3 into the autophagic membranes via dynein-mediated transport.

Evaluation of autophagy activity of mutant Merlin (Aim 2)

2a. Preparation of expression constructs

Studies on human NF2 revealed single amino acid mutations at >70 amino acids across the entire stretch of Merlin sequence. To determine the relevance of Merlin's role in autophagy with respect to NF2 pathogenesis, we introduced a series of representative Merlin mutations into mammalian expression constructs, and tested for their ability to induce autophagy.

2b. Transfection and immunocytochemistry experiments

We first evaluated the $Lys^{72}$->$Glu^{72}$ (K79E) mutant and the $Glu^{270}$->$Gly^{270}$ (E270G) mutant in IP experiments. K79E is shown to abolish the interaction with dynein, and thus expected to impair the role of Merlin in vesicle trafficking. E270G is shown to cause hyperproliferation in cells, and thus considered to represent tumor suppressor activity of Merlin. K79E lost the ability to interact with LC3 (**Figure 7a**), whereas E270G showed attenuated levels of interaction with LC3 (**Figure 7b**).

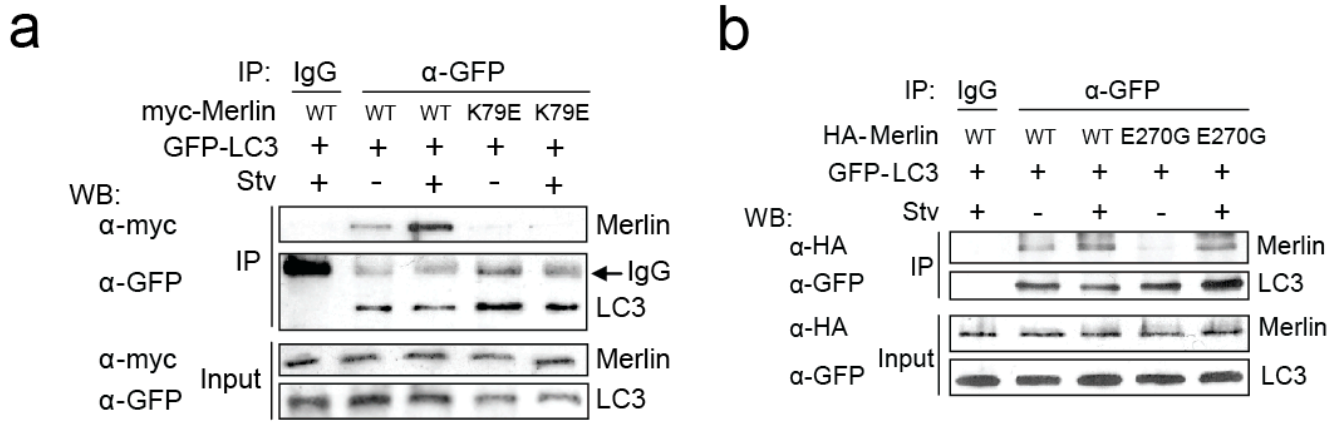
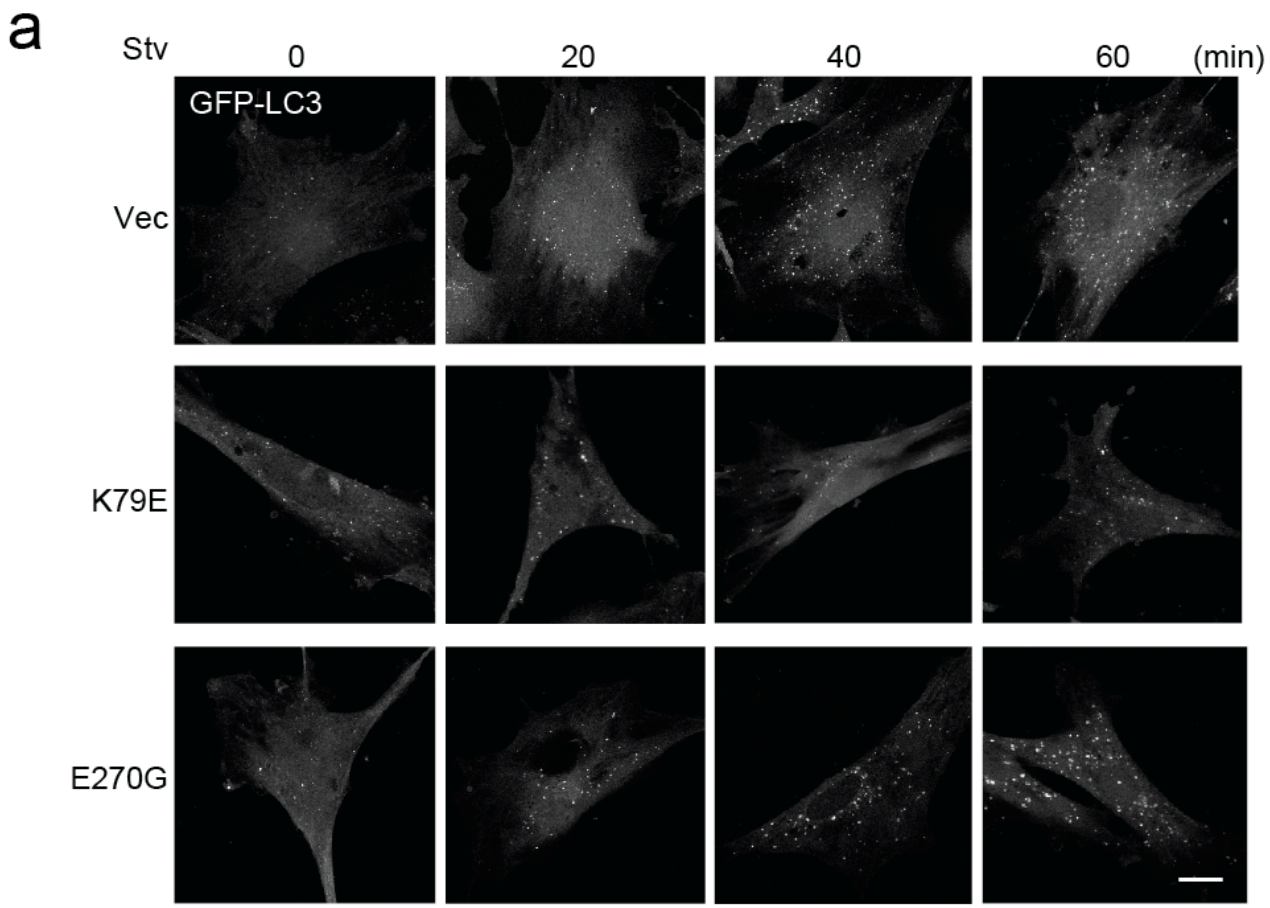



**Figure 7. Merlin$^{K79E}$ and Merlin$^{E270G}$ show differential affinity to LC3**
HEK293T cells were transfected with the indicated plasmids and the resulting cell lysates were analyzed by immunoprecipitation (IP) with anti-GFP (α-GFP) or the control IgG, followed by Western blots (WB) using the indicated antibodies.

To evaluate the autophagy-inducing ability of these mutants, the expression constructs for Merlin K79E and E270G mutants were transfected to MEFs expressing GFP-LC3 (prepared above in Aim 1e), and numbers of GFP-LC3-positive puncta (autophagic membrane) were scored upon autophagy induction. K79E showed significantly lower levels of puncta formation than control, and E270G showed levels higher than K79E and lower than control (**Figure 8**).



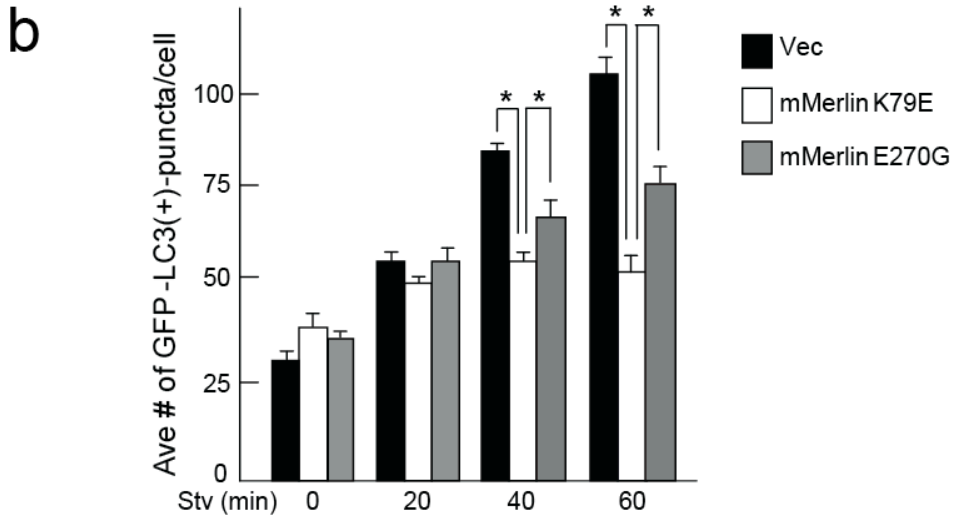
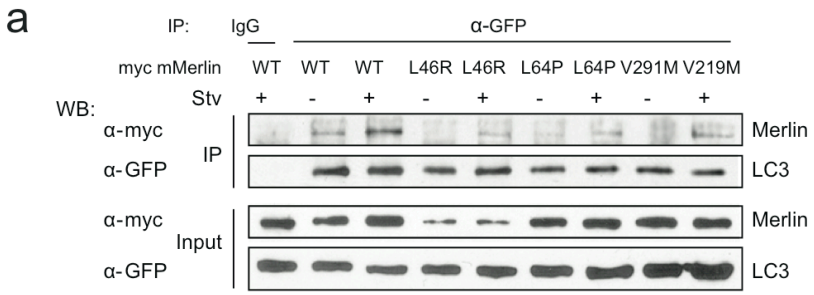
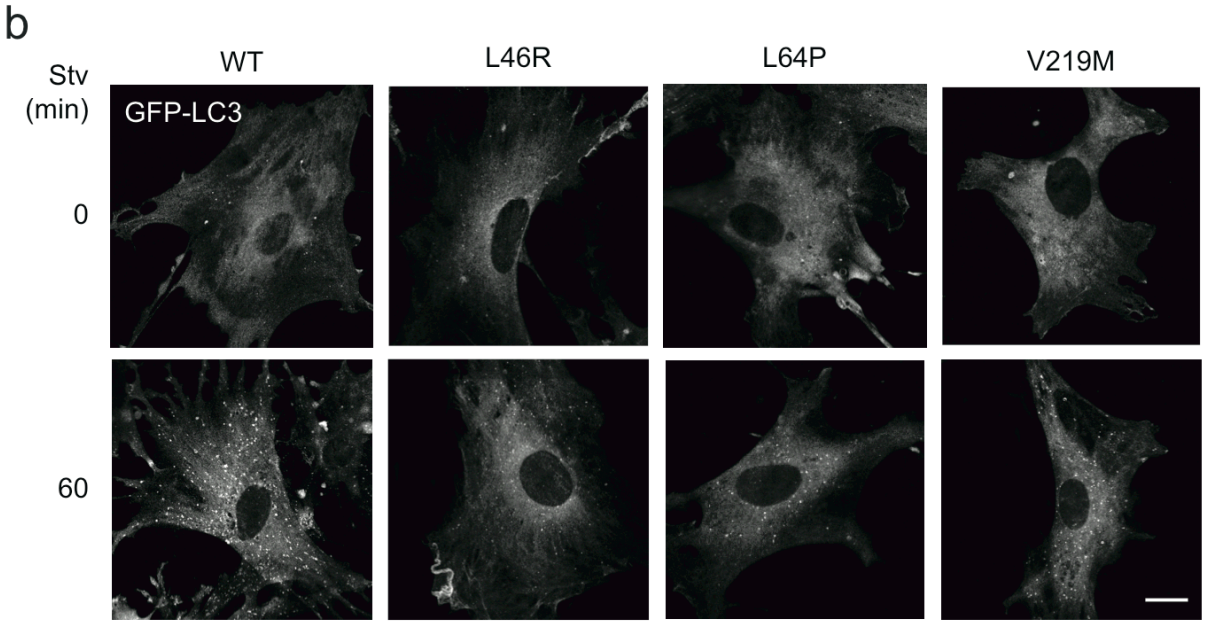



**Figure 8. Merlin$^{K79E}$ and Merlin$^{E270G}$ show differential ability to induce autophagy**
**(a)** MEFs expressing GFP-LC3 were transfected with the indicated plasmids and observed by confocal microscopy. Representative images 0, 20, 40, and 60 min after autophagy induction were shown. Scale bar 10μm.
**(b)** Quantitative analysis of immunocytochemistry in **(a)**. Average numbers of puncta were scored for >30 cells per each condition.

To further determine the relevance of Merlin-mediated autophagy in NF2 pathogenesis, we introduced additional mutations into Merlin expression constructs, and tested for their ability to interact with LC3 and to induce autophagy. Lys$^{46}$->Arg$^{46}$ (L46R), Lys$^{64}$->Pro$^{64}$ (L64P), and Val$^{219}$->Met$^{219}$ (V219M) mutants all showed attenuated affinity to LC3 before and after autophagy induction, when compared with wild-type Merlin (**Figure 9a**).



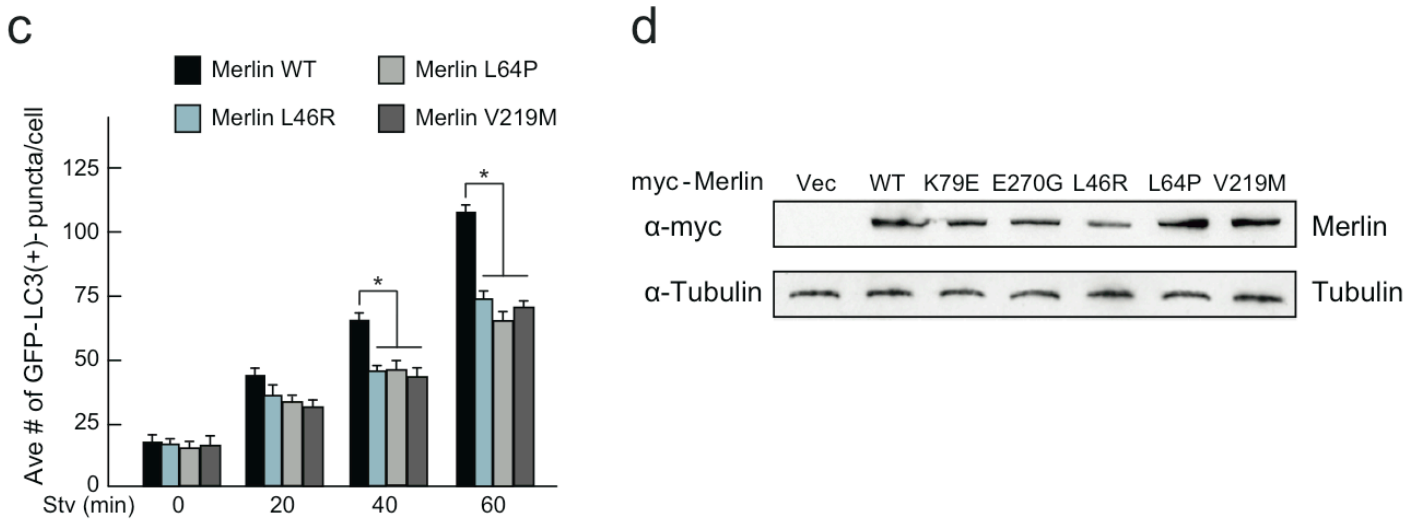



**Figure 9. Merlin point mutants show weak affinity to LC3 and lower levels of autophagy induction**
**(a)** HEK293T cells were transfected with the indicated plasmids and the resulting cell lysates were analyzed by immunoprecipitation (IP) with anti-GFP (α-GFP) or the control IgG, followed by Western blots (WB) using the indicated antibodies.
**(b)** MEFs expressing GFP-LC3 were infected with the lentivirus expressing the indicated Merlin mutants and observed by confocal microscopy. Representative images at the fed condition and 60 min after autophagy induction were shown. Scale bar 10μm.
**(c)** Quantitative analysis of immunocytochemistry in **(b)**. Average numbers of puncta were scored for >30 cells per each condition.
**(d)** MEFs that stably expressed each Merlin mutant were analyzed by WB to evaluate the level of Merlin expression.

In addition, the autophagy-inducing abilities of these mutants were evaluated. MEFs expressing GFP-LC3 were transduced with the lentiviruses expressing each Merlin mutants (L46R, L64P, V219M), and numbers of GFP-LC3-positive puncta were scored upon autophagy induction. All these mutants showed significantly lower levels of puncta formation than Merlin WT control (**Figure 9b-d**).

2c. Data analysis for 2a and 2b

The results of LC3 coupling efficiency, as well as autophagy induction analyses (**Figures 7-9**), suggest that the role of Merlin in autophagy has general relevance to NF2 pathogenesis.

Evaluation of autophagy inhibitors in 3D culture system (Aim 3)

3a. Preparation of MCF10A cells

We established the three-dimensional (3D) culture system using MCF10A cells. In brief, MCF10A cells were first maintained in monolayer cultures in growth media (DMEM/F12 plus 5% horse serum, 10μg/ml insulin, 20ng/ml EGF, 100ng/ml choleratoxin, 0.5ug/ml hydrocortisone, 100U/ml penicillin, and 100mg/ml streptomycin, then the 3D cultures of MCF10A were prepared on Matrigel in growth media lacking epidermal growth factor (EGF) and with reduced horse serum (2%). To induce autophagy, amino acid-free starvation media (Earle's balanced salt solution), or complete media with rapamycin (100nM) were used. Autophagy inhibitors used were: 3-methyladenine (10mM) and bafilomycin A1 (50nM).

3b. Initiate 3D cultures

MCF10A 3D cultures were prepared by seeding $1x10^4$ MCF10A cells from the monolayer culture onto a Matrigel-coated culture chamber slide. Under this condition, single cell of MCF10A would develop into a sphere-like structure called acini within 10 to 14 days *in vitro*. Genetic manipulation, such as Merlin knockdown or Ulk1/Atg1 expression, can be accomplished by infecting MCF10A cells with a lentivirus encoding Merlin-knockdown shRNA or with a retrovirus expressing Ulk1, respectively, at the beginning of the culture when cells are at the single cell stage, and the transduced cells were selected by puromycin selection (2μg/ml) for 3 days.

3c. Drug treatment of MCF10A 3D cultures

MCF10A cell 3D cultures on Matrigel were treated with a series of autophagy inducers or inhibitors for the last 6 days of the culture period, to evaluated cellular response (*i.e.*, the extent of metabolic stress as estimated by DNA damage response (phospho-histone 2A.Ser-139 [pH2A.X.] or by hypoxia marker [hpi]), cell growth (Ki-67 proliferation marker immuno-staining), or a cell-shape marker (β-catenin antibody)) (**Figure 10**).

3d. Imaging analysis by microscopy

These data demonstrated that loss of Merlin resulted in higher levels of cellular proliferation (higher Ki-67), as well as higher levels of metabolic stress, as evidenced by higher hpi staining (**Figure 10**) and pH2A.X staining (**Figure 11**), as compared with control knockdown cells (Ct-KD). Rapamycin (Rapa) treatment significantly suppressed cellular proliferation and the degree of metabolic stress.

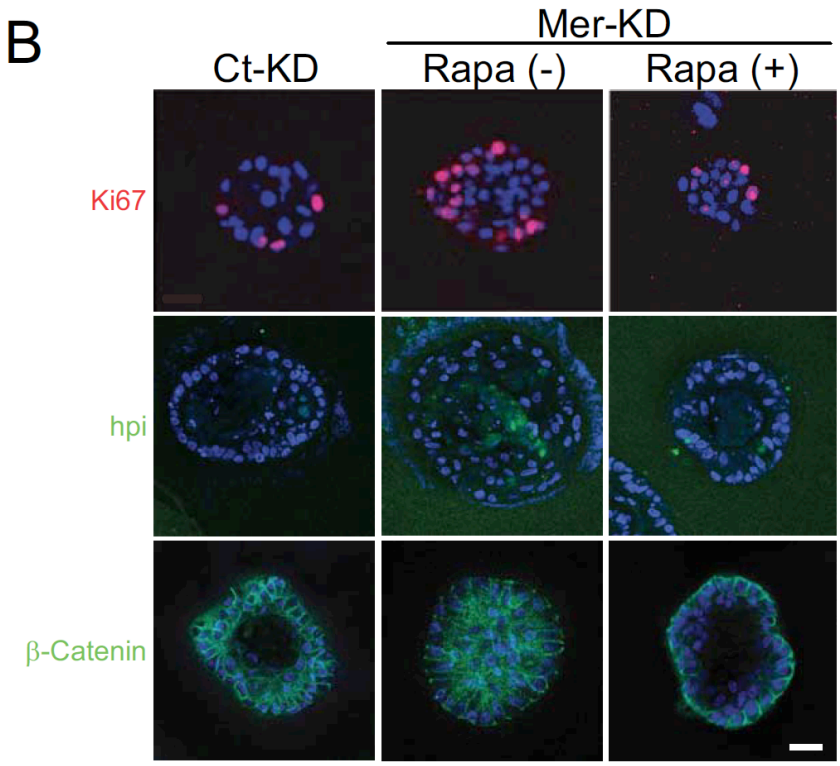



**Figure 10. Merlin KD led to higher metabolic stress in MCF10A 3D culture system**
MCF10A cells were infected with the lentivirus encoding the control (Ct-KD) or Merlin knockdown (Mer-KD) shRNA. Single MCF10A cells were allowed to grow as a 3D sphere on Matrigel. The cultures were treated with rapamycin (Rapa) for the last 6 days in culture, fixed and stained with anti-Ki67 (red) at 12DIV, anti-Hypoxyprobe-1 (green) at 14DIV, or anti-β-catenin antibodies (green) at 10DIV, as well as with DAPI (blue). Each sphere was observed by confocal microscopy and the optical section through the mid-plane of the sphere is shown. Scale Bar: 40μm

To further determine the extent to which autophagy manipulation could influence metabolic stress seen in Merlin-KD 3D cultures, autophagy was either upregulated by Ulk1/wt, or downregulated by Ulk1/dn expression, or pharmacologically inhibited by bafilomycin A1 or 3-methyladenine (3MA). Enhancing autophagy reduced the level of metabolic stress (*i.e.*, pH2A.X), whereas inhibition of autophagy enhanced metabolic stress in 3D cultures (**Figure 11**).

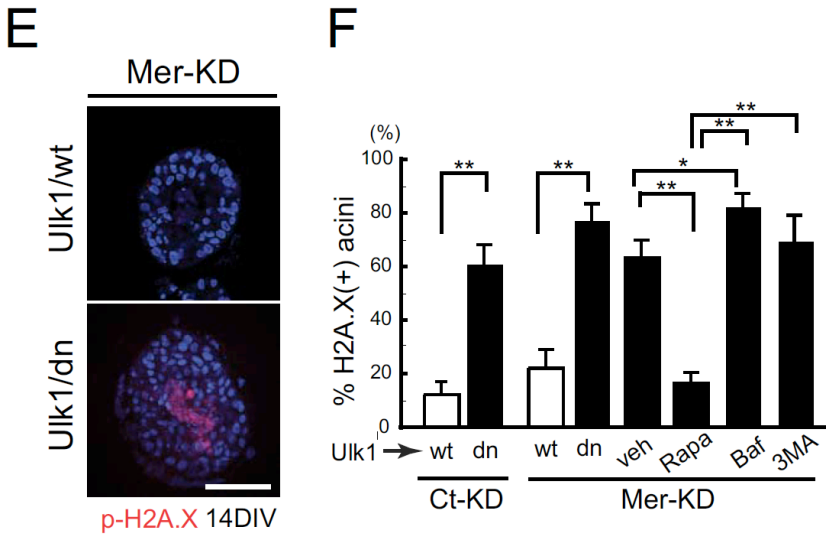



**Figure 11. Autophagy induction led to reduced levels of metabolic stress in Merlin-KD cells**
MCF10A 3D cultures were infected with the control (Ct-KD) or Merlin-knockdown (KD) lentivirus, as well as with the retrovirus expressing either wild-type Ulk1 (Ulk1/wt) or dominant-negative Ulk1 (Ulk1/dn), treated with autophagy inducers/inhibitors as indicated, and stained at 14DIV with anti-pH2A.X (red) and DAPI (blue). Scale bar: 40μm
Graph: Quantitative analysis of immunocytochemistry. MCF10A spheres were treated with rapamycin (Rapa), bafilomycin A1 (Baf), 3-methyladenine (3MA), or vehicle (Veh), and the rate of spheres with pH2A.X-positive cells (%) were scored (mean ± SEM). *; $p<0.05$, **; $p<0.005$; Student's *t*-test

3e. Data analysis for 3d

The data demonstrated that loss of Merlin resulted in higher levels of cellular proliferation (higher Ki-67), as well as higher levels of metabolic stress, as compared with control cells. Autophgay induction by rapamycin treatment or Ulk1 expression significantly suppressed the degree of metabolic stress, which is recently shown to promote tumorigenesis. Thus, the results shown in **Figures 10-11** are consistent with the role of autophagy in tumor suppression, and suggest that activation of autophagy pathway could serve as a therapeutic strategy against tumors in which Merlin-mediated autophagic activity is lost or attenuated.

Preparation of research papers (Aim 4)

4a. Data analysis (Aim 1, 2, 3)

Data shown in Aim 1 through 3 were analyzed in the last year of the grant period.

4b. Assembly of analyzed data

These data were assembled into a paper.

4c. Submitting the manuscripts that describe the results of the proposed research

The manuscript has been submitted and now under revision step.

**Key Research Accomplishments:**

- Merlin promotes the accumulation of the LC3 autophagy-related protein on the autophagic membrane precursor called isolation membrane, by serving as a linker between LC3 and dynein motors.

- NF2-associated mutation Merlin$^{K79E}$ specifically inhibits autophagy induction without affecting its role as a growth suppressor, while the additional mutation, Merlin$^{E270G}$, affects not only the growth suppressive function of Merlin but also attenuated autophagy.

- Additional NF2-associated Merlin mutations affected autophagy to a varying degree, suggesting that the role of Merlin in autophagy is relevant to NF2 pathogenesis.

- Attenuated autophagy caused by loss of Merlin led to increased levels of metabolic stress, which can be mitigated by autophagy inducer rapamycin.

**Reportable Outcomes:**

- The above data was presented at the poster session at Childrens' Tumor Foundation (CTF) annual Meeting held at Jackson Hole WY (June 2011).

- Dr. Donald Jhung Jr. obtained Ph.D. as a result of his studies supported by this award (June 2011).

- CTF-drug discovery initiative research award was obtained by the PI, based on part of the results obtained by this award (Oct. 2011).

- Research associate, Ms. Yuki Hirota, was employed (June 2011~March 2014) and contributed to the majority of the results shown above.

- Poster session describing this work presented by Ms. Hirota was selected No. 1 in the annual poster session presentation competition held at City of Hope (Jan. 2012).

- PI presented this work at the Special symposium on Autophagy; Health and Disease, held at City of Hope (Mar. 2012).

- PI presented this work at the oral session at Childrens' Tumor Foundation (CTF) annual Meeting held at New Orleans (June 2012).

- Dr. Akiko Sumitomo was hired (June 2012).

- This work was presented by Ms. Hirota at the annual poster session held at City of Hope (Nov. 2012).

- PI presented this work at the 6$^{th}$ International Symposium on Autophagy held at Okinawa (Nov. 2012).

- The research paper describing part of the above results was submitted to a journal in Sep. 2013, and has been under revision to be considered for publication as of June 2014.

**Conclusion:**

We successfully confirmed the novel role of Merlin in promoting autophagy. We showed that Merlin is a part of multi-protein complex that serves as a scaffolding machinery to promote autophagic membrane assembly. At least one of the NF2-associated mutation ($Merlin^{K79E}$) was found inhibitory to autophagy induction without showing the effect on cellular proliferation, suggesting that the role of Merlin in proliferation control vs. autophagy regulation could be separable. Additional mutations in Merlin (E270G, L46R, L64P, and V219M) also attenuated the ability of Merlin to induce autophagy. This result demonstrates the dual role of Merlin tumor suppressor in growth suppression and autophagy induction, both of which are relevant to NF2-related tumorigenesis. Further analyses are needed to delineate the role of Merlin in autophagy.

**References:**

None

**Appendices:**

None

**Supporting Data:**

Eleven figures and one schematic diagram are included in the body of this report.